[CANCER RESEARCH 55, 1211–1214, March 15, 1995]

Advances in Brief

$p27^{Kip1}$: Chromosomal Mapping to 12p12–12p13.1 and Absence of Mutations in Human Tumors[1]

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Abstract

The $p27^{Kip1}$ gene codes for a cyclin-dependent kinase inhibitor implicated in G_1 arrest by transforming growth factor β, cell-cell contact, agents that elevate cyclic AMP, and the growth-inhibitory drug rapamycin. *p27* binds to and inhibits complexes formed by cyclin E-cdk2, cyclin A-cdk2, and cyclin D-cdk4. The involvement of *p27* in the negative regulation of cell proliferation suggests that it may also function as a tumor suppressor gene. Using a combination of somatic cell hybrid panels and fluorescence *in situ* hybridization $p27^{Kip1}$ has been mapped to the short arm of chromosome 12 at the 12p12–12p13.1 boundary, reported to harbor deletions and rearrangements in leukemia and mesotheliomas. In order to assess potential $p27^{Kip1}$ gene alterations, we have screened a total of 147 human primary solid tumors and found no detectable cancer-specific mutations. These results argue that the often observed loss of antimitogenic transforming growth factor β responsiveness in human cancer cells is not due to structural defects in $p27^{Kip1}$.

Introduction

Neoplastic diseases are characterized by an uncoordinated cell growth (1). Cellular proliferation follows an orderly progression through the cell cycle, which is controlled by protein complexes composed of cyclins and cdk[3] (2, 3). The search for molecular aberrations modifying these crucial regulators in human tumors revealed that cyclin D1 at chromosome 11q13 is amplified in a subset of cancers, acting as an activated oncogene (4, 5). Similarly, cdk4 is overexpressed in certain tumors due to its coamplification as a component of an amplicon on the long arm of chromosome 12, where it maps (6). More recently, a family of negative cell cycle regulators has been identified that function as cdk inhibitors (7). One of these proteins is p21 (also known as WAF1, Cip1, Sdi1, and CAP20), the gene of which maps to 6p21.1 and inhibits cyclin E-cdk2, cyclin A-cdk2, and cyclin D-cdk4 complexes (8–10). A potential role for p21 in tumorigenesis was postulated on the basis of its transcriptional control by *p53* (11, 12). However, no molecular alterations of p21 have been described to date. Two other cdk inhibitors are *p16* (INK4A, MTS1, or Pic2) (13–15) and *p15* (INK4B or MTS2) (16), which form binary complexes with *cdk4* or its isoform *cdk6* (13, 16). These two genes map to 9p21, a region that accounts for loss of heterozygosity and homozygous deletions in various human tumor types, including melanoma and bladder carcinoma (17, 18). *p16* point mutations are frequent in esophageal carcinomas and melanomas (19, 20), and selective deletion or mutation of *p15* and *p16* is observed at high frequency in tumor-derived cell lines (14, 17).

To this rapidly growing group of cdk inhibitors a new member has been added, $p27^{Kip1}$ (21–23). *p27* is a negative regulator implicated in G_1 arrest by TGFβ, cell-cell contact, agents that elevate cyclic AMP, and the growth-inhibitory drug rapamycin (21–25). *p27* associates with cyclin E-cdk2, cyclin A-cdk2, and cyclin D-cdk4 complexes, abrogating their activity (21–23). Acting as a stoichiometric inhibitor of G_1 cyclin-cdk complexes, excess *p27* interferes with G_1 progression when its levels are increased by cyclic AMP agonists in macrophages (24) or by rapamycin in T lymphocytes (25), or when cyclin-cdk levels are decreased by TGFβ or cell contact in lung epithelial cells (22). Loss of antimitogenic responsiveness to antimitogenic agents, in particular TGFβ, is often observed in human cancer (26). In order to assess potential $p27^{Kip1}$ gene alterations and their prevalence in human cancer, we have examined this gene in a variety of fresh, noncultured, primary and metastatic tumor tissues. We have also mapped $p27^{Kip1}$ to the 12p12–12p13.1 boundary using a combination of somatic cell hybrid panels and fluorescence *in situ* hybridization.

Materials and Methods

Source of Tissue. The tumor tissues analyzed included bladder carcinomas ($n = 15$), prostatic carcinomas ($n = 20$), renal carcinomas ($n = 17$), pancreatic adenocarcinomas ($n = 21$), breast carcinomas ($n = 6$), lung carcinomas ($n = 11$), germ cell tumors ($n = 10$), melanomas ($n = 27$), and soft tissue sarcomas ($n = 20$). Adjacent tumor and normal tissues from all cases, with the exception of 20 melanoma cases in which only tumor tissue was available, were obtained from surgical specimens processed in the Department of Pathology, Memorial Sloan-Kettering Cancer Center, New York, NY. Samples were embedded in cryopreservative solution (OCT Compound; Miles Laboratories, Elkhart, IN), snap-frozen in isopentane precooled in liquid nitrogen, and stored at −70°C. Representative hematoxylin-eosin-stained sections of each frozen block were examined microscopically to confirm the presence of tumor, as well as to evaluate the percentage of tumor cells comprising these lesions and the extent of tumor necrosis. In addition, 15 cell lines derived from male germ cell tumors were also analyzed in this study.

Southern Blotting Analysis. A 0.6-kilobase cDNA fragment from human $p27^{Kip1}$ was used as a probe in Southern blots to assess deletions. As a control, a cDNA fragment probe from the *GAPDH* gene was used. Southern analysis was performed as described (27). Briefly, DNA was extracted by the nonorganic method (Oncor, Gaithersburg, MD) from paired normal and tumor samples, digested with *Hind*III restriction enzyme, electrophoresed in 0.7% agarose gels, and blotted onto nylon membranes. The membranes were prehybridized with Hybrisol I (Oncor) at 42°C for 2 h and hybridized overnight at 42°C with probes labeled to high specific activity with [^{32}P]dCTP. Membranes were then washed at high stringency and subjected to autoradiography using intensifying screens at −70°C for 24–72 h.

SSCP Analysis and DNA Sequencing. These assays were performed according to a slight modification (28) of the method reported by Orita *et al.* (29). The four sets of primers used to amplify the entire coding region of the

Received 12/1/94; accepted 2/1/95.

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[1] This research was supported in part by NIH Grants CA-47538, CA-47179, CA-58514, and CA-DK-47650 (C. C-C.). J. M. is a Howard Hughes Medical Institute Investigator, and support for this work was provided in part by the Howard Hughes Medical Institute.

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[3] The abbreviations used are: cdk, cyclin-dependent kinases; TGFβ, transforming growth factor β; PCR-SSCP single-strand conformation polymorphism.

human $p27^{Kipl}$ and the size of PCR products generated by them are illustrated in Fig. 1 (see figure legend for the sequence of these primers). DNA was amplified following 30 cycles of PCR [30 s at 94°C; 30 s at 62°C (set I), 62°C (set II), 60°C (set III), 65°C (set IV); 1 min at 72°C] using a thermal cycler (Perkin-Elmer Cetus, Foster City, CA). Amplified samples were then denatured and loaded onto a nondenaturing polyacrylamide gel containing 10% glycerol and run at room temperature for 12–16 h at 10–12 W. Gels were dried and exposed to X-ray film at −70°C for 4–16 h.

Amplification of genomic DNA for direct sequencing was independent of that used for SSCP analysis. DNA fragments were purified from 1.5% regular agarose gels and sequenced by the dideoxy method (30). Both strands were sequenced and DNAs from normal control samples were sequenced in parallel to confirm mutations as well as to exclude polymorphisms and thermal-stable DNA polymerase misincorporations.

Somatic Cell Hybrid Analysis. The 0.6-kilobase cDNA insert from $p27^{kip1}$ probe was labeled with [^{32}P]dCTP and hybridized with Southern blots containing a panel of *Pst*I restriction-digested somatic cell hybrid DNAs derived from 27 cell lines containing various combinations of human chromosomes against a hamster background (BIOS, New Haven, CT). PCR analysis of the above panel of somatic cell hybrid DNAs and additional DNAs from hybrid cell lines containing only chromosome 12 and only 12p (M28) (provided by Peter Marynen, Belgium) was performed using the primers designed to amplify a 224-base pair genomic fragment.

Fluorescence *in Situ* Hybridization. Two MEGA YACs (738b11 and 954g10) positive for $p27^{kip1}$ by PCR analysis were labeled with biotin-11-dUTP using a bio-nick labeling system (Life Technologies, Grand Island, NY). Preparation of metaphase chromosomes from phytohemagglutinin-stimulated and 5-bromodeoxyuridine-synchronized lymphocyte cultures, *in situ* hybridization, and detection of signals by indirect immunofluorescence were performed as described previously (31). Separate images of 4,6-diamidino-2-phenylindole-counterstained chromosomes and hybridization signals were captured by a cooled carge-coupled device camera (Photometrics, Tucson, AZ), and analyzed using a Smartcapture Imaging System (Imagenetics, Framingham, MA).

Results and Discussion

Discrepancies of reported results aimed at the identification of mutations in primary, noncultured tumor lesions may be explained by the use of distinct probes and methods in different settings. Furthermore, the *bona fide* presence of tumor, the normal:tumor ratio, the extent of tumor necrosis, and the stage and grade of the lesions being studied are rarely reported. We have followed a methodology whereby from a single tissue sample we can perform different techniques in search of the phenotype and genotype of tumor cells in that specimen (29). Briefly, representative hematoxylin-eosin-stained sections of each normal and tumor fresh frozen blocks are microscopically evaluated to determine the presence and characteristics of the tumor. In several instances, microdissection is performed to restrict normal cell contamination to a minimum. This is of utmost importance when performing molecular genetic techniques on surgical specimens aimed at the detection of deletions. In addition, comparative analyses of normal *versus* tumor tissue pairs from the same individual are performed to identify possible polymorphisms.

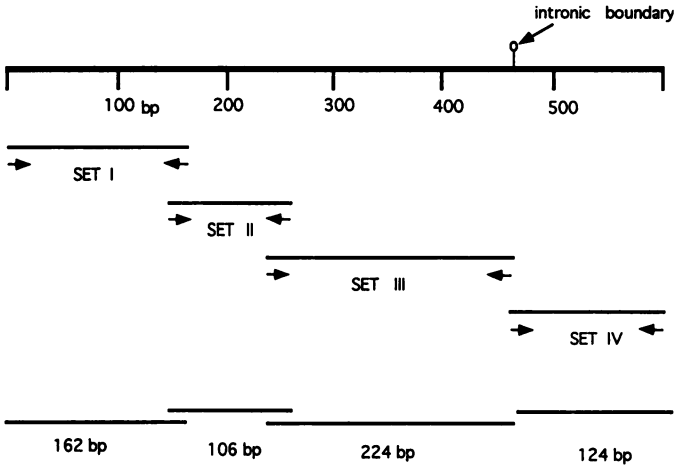


Fig. 1. Strategy for the analysis of $p27^{Kip1}$ using PCR-SSCP. The sequences of primers for each set utilized are:

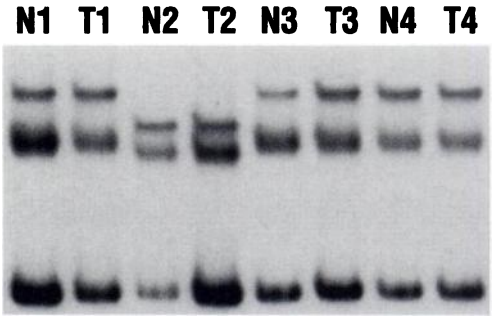
Set I: 5′ATGTCAAACGTGCGAGTGTC3′, 5′CTCTTCCATGTCTCTGCAGTG3′;

Set II: 5′CACTGCAGAGACATGGAAGAG3′, 5′TCTGTAGTAGAACTCGGGCAA3′;

Set III: 5′TTGCCCGAGTTCTACTACAGA3′, 5′AGGGTCATTACCGTCGGTTGC3′;

Set IV: 5′CCCCTGCGCTTAGATTCTTCT3′, 5′CGTTTGACGTCTTCTGAGGCC3′.

bp, base pairs.



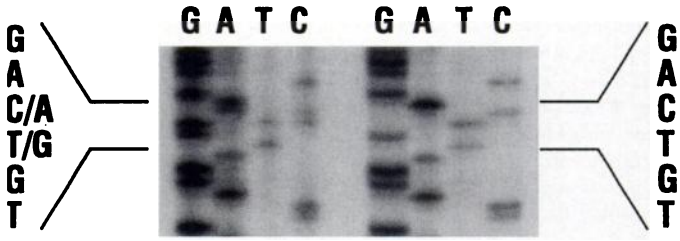


Fig. 2. Polymorphisms detected by single-strand conformation polymorphism analysis of $p27^{Kip1}$. Direct sequencing revealed a T to G substitution at base 326, changing a nonconserved valine to glycine. *N1–N4* and *T1–T4*, normal and tumor tissues, respectively.

The present survey has used the above mentioned strategy and has characterized a total of 147 solid tumors, including carcinomas, germ cell tumors, sarcomas, and melanomas, in search of potential $p27^{Kip1}$ mutations. In order to evaluate deletions, Southern blot analyses were performed on DNA extracted from paired normal and tumor samples. In addition, to examine for structural alterations of $p27^{Kip1}$, aliquots of extracted DNA from all samples were analyzed using PCR-SSCP and sequencing assays. Fig. 1 illustrates the screening strategy followed and the sets of primers utilized to amplify the entire human $p27^{Kip1}$ coding region.

Comparing tumor *versus* normal tissue signal intensities, no deletions of the $p27^{Kip1}$ locus were identified. Point mutations were not detected in the tumors analyzed, as assessed by the lack of shifts in mobility in tumor lanes relative to normal tissue lanes in PCR-SSCP gels. A polymorphism at base 326 (T to G), changing a nonconserved valine to glycine, was observed in approximately 11% of the cases studied (Fig. 2). Furthermore, analysis of germ cell tumor cell lines, known to contain an isochromosome for the short arm of chromosome 12 [i(12p)], did not revealed any deletions or point mutations of $p27^{Kip1}$ (data not shown).

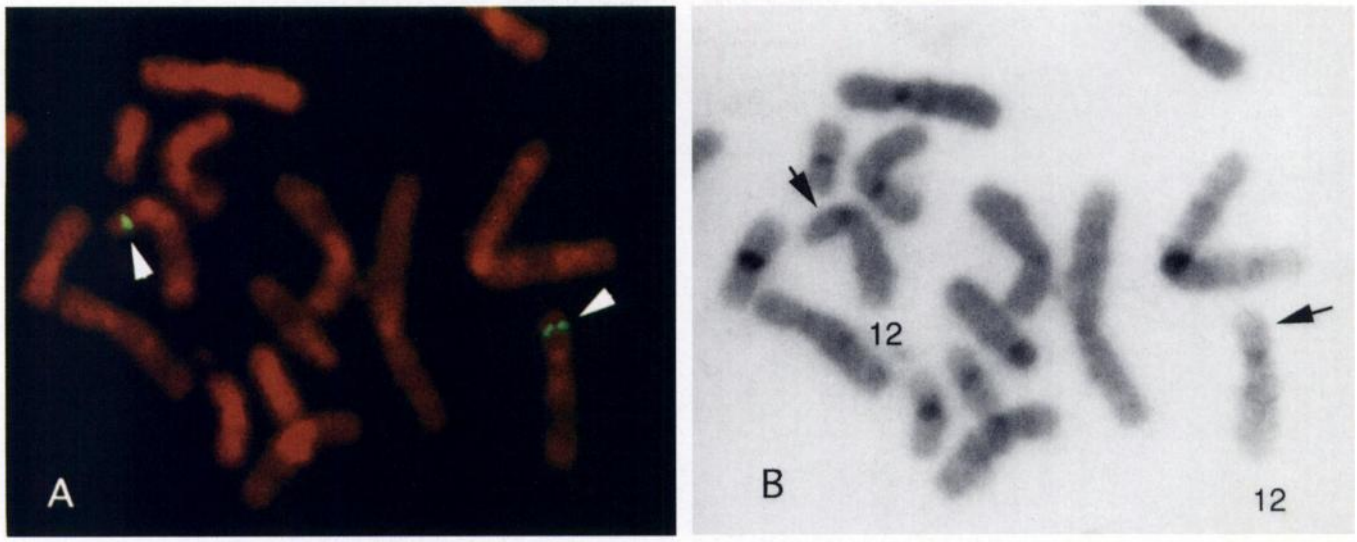


Fig. 3. Subregional localization of $p27^{kip1}$ to 12p12–12p13.1 by fluorescence *in situ* hybridization. Partial metaphase stained with propidium iodide (*A*) and stained for G-banding pattern (*B*), following hybridization of YAC 738b11. *Arrowheads*, hydridization signals; *arrows*, band 12p12.

Southern blot analyses of the somatic cell hybrid panel DNAs hybridized with the full-length cDNA probe showed signals syntenic to chromosome 12 with 100% concordance. PCR analysis using the primer set III on DNAs from the same panel of somatic cell hybrids, and additional somatic cell hybrid cell lines containing chromosome 12 and 12p alone, suggested the localization of $p27^{Kip1}$ to the short arm of chromosome 12 (data not shown). Two MEGA YAC clones containing $p27^{Kip1}$ gene were identified by PCR on YAC pools from 12p regions (data not shown). In order to subregionally map the gene, we used fluorescence *in situ* hybridization by hybridizing the two YAC DNAs to normal human metaphase chromosomes. Analysis of hybridization signals showed specific clustering at 12p, allowing the precise localization of the $p27^{Kip1}$ gene to the 12p12–12p13.1 boundary (Fig. 3).

The 12p12–12p13.1 region to which $p27^{Kip1}$ maps is of interest in relation to cancer. Deletions and rearrangements of 12p have been reported to occur frequently in leukemia (32) and in peritoneal mesothelioma (33). Cytogenetic and molecular genetic analyses of male germ cell tumors have identified a number of nonrandom abnormalities affecting chromosome 12. The most specific of these alterations is i(12p), which has been shown in over 85% of germ cell tumors (34). In addition, i(12p) has been also reported in ovarian teratoma (35) and malignant ovarian neoplasms (36). More recently, introduction of a portion of chromosome 12 (12pter–12q13) into a human prostate cancer cell line led to the complete suppression of tumorigenicity in athymic nude mice (37).

Although the role of *p27* in negative regulation of cell proliferation suggests that it may also function as a growth suppressor gene, the absence of detectable genetic abnormalities of $p27^{Kip1}$ in the tumors examined argues that it is not a frequent target of mutations predisposing to cancer. Pietenpol *et al.* (38) and Bullrich *et al.* (39) have independently noted the absence of $p27^{Kip1}$ mutations in a variety of tumors analyzed. However, *p27* acts as a stoichiometric inhibitor of G_1 cyclin-cdks and even modest changes in the relative levels of *p27* can have a major effect on G_1 progression (24). Therefore, it is conceivable that posttranscriptional or posttranslational modifications, or even altered patterns of *p27* expression, may result as a part of the processes of tumorigenesis or tumor progression. Alternatively, other genes and their encoded products upstream or downstream of the *p27* pathway of signaling may be the ones affected in certain human cancers. Our study was not designed to determine such alterations and more detailed analyses will be required in order to examine these possibilities.

Acknowledgments

We thank Drs. Raju Chaganti and Vundavalli Murty for their assistance with cytogenetic analysis and critical review of the manuscript.

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Cancer Res 1995;55:1211-1214.